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Original Article

Neratinib inhibits the malignant phenotype via inhibiting the HER2 in osteosarcoma cells

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Abstract: Osteosarcoma is a deadly bone tumor. Our former study found that inhibiting autophosphorylation of HER2 could restrain the invasion and migration ability of osteosarcoma cells. As a pan-HER inhibitor, Neratinib, has completed preclinical phases and is currently undergoing various clinical trials. The objective of this study was to confirm the efficacy of Neratinib in the treatment of HER2 amplified osteosarcoma. Migration, invasion and proliferation abilities of OS cells were tested by wound healing, transwell invasion and CCK-8 assays. Results revealed that the migration, invasion and proliferation properties were declined in cells treated with Neratinib ($P<0.05$). Furthermore, in order to investigate the possible molecular mechanisms involved, western blot assay was used to determine the expression of p-HER2, phosphatidylinositol 3-kinase (PI3K), p-AKT and AKT proteins in OS cells. Results showed that the expression level of p-HER2, PI3K and p-AKT were decreased after treatment with Neratinib ($P<0.05$). Collectively our findings identified the tumor suppressive role of Neratinib in the development of malignant human osteosarcoma, and suggest its potential as a functional biomarker with predictive value in cancer predisposition, progression and treatment in clinic.

Keywords: Osteosarcoma, Neratinib, HER2, malignant phenotype, PI3K/AKT signaling pathway

Introduction

Osteosarcoma (OS) is the most common malignant bone tumor in adolescents, and the survival rate is less than 10-15% [1]. In recent years, the treatment for osteosarcoma has been updated slowly; but the five-year survival rate still lingers in recent decades [2]. Pulmonary metastasis is the primary cause of death among the patients suffering from OS [3]. It is difficult to establish a target for OS prevention and treatment owing to the undefined molecular mechanism. Therefore, exploring the molecular mechanisms of OS cells invasion, migration and proliferation are urgently needed.

Human epidermal growth factor receptor 2 (HER2) is a transmembrane receptor tyrosine kinase encoded by the oncogene ErbB2/HER2/neu [4], geared to the epidermal growth factor receptor (EGFR) family. Overexpression of HER2 has been found in varieties of cancers such as breast cancer [5], ovarian cancer [6] and OS [7]. In addition, HER2 is a major signal protein associated with the malignant phenotype of osteosarcoma cells [8, 9], and the phosphorylated HER2 plays a crucial role in cell signal transduction through the intracellular PI3K/Akt signal pathway [10].

The HER (ErbB) receptor tyrosine kinase receptors have been referred to many cancers and several anti-HER treatments are now undergoing clinical application. In recent years, a new group of compounds that combined irreversibly with the adenosine triphosphate binding pocket of HER receptors have been produced. Neratinib, one of these compounds, its molecular formula is $C_{30}H_{29}ClN_6O_3$. Neratinib is an irreversible, small-molecule, tyrosine kinase inhibitor of both EGFR and HER-2, acting at the ATP binding sites of their tyrosine kinase domains [11]. Neratinib selectively inhibit HER-1 and

HER-2 in the EGFR family [11]. It is in phase III clinical trials, combined with Capecitabine in the treatment of HER-2 positive advanced breast cancer.

Nevertheless, the function of Neratinib on the malignant phenotype potential of HER2 and the molecular mechanisms in OS cells need to be further explored. To explore the effect of Neratinib on osteosarcoma and to clarify its potential molecular mechanisms, the present research was conducted by evaluating the effect of Neratinib on human U2-OS and 143B cell malignant phenotypes *in vitro.*

Materials and methods

Cell lines and cell culture

The U2-OS and 143B cell lines were purchased from American Type Culture Collection (Manassas, VA, USA), and were respectively cultured in Roswell Park Memorial Institute (RPMI-1640) and Dulbecco's modified Eagle's medium (DMEM) (HyClone, Waltham, MA, USA), supplemented with 10% fetal bovine serum (FBS) with 100 U/ml penicillin and 100 U/ml streptomycin. Cells were cultured at 37°C in 5% CO_2.

CCK8 assay

The U2-OS and 143B cells were seeded out in 96-well tissue culture plates in the minimum essential medium. The next morning, we replaced nutrition solution with the medium of different concentrations of Neratinib (1, 2, 3, 4 and 5 μmol/L) (Abmole BioScience). Then, 10 μL of CCK8 solution was added to each well, and the plates were incubated for an additional 2 h at 37°C. Cell viability was measured as the absorbance at 450 nm with a microplate reader. The mean optical density (OD) values from triplicate wells for each treatment were used as the index of cell viability.

Migration assay

In brief, cells were grown to confluence in six-well tissue culture plastic dishes to a density of approximately 5×10^6 cells/well and afterward treated with Neratinib (2.5 μmol/L for U2-OS and 3 μmol/L for 143B cells) for 24 h. The cells were denuded by dragging a rubber policeman (Fisher Scientific, Hampton, NH, USA) through the center of the plate. Cultures were rinsed with PBS and replaced with fresh quiescent medium alone or containing 10% FBS, following which the cells were incubated at 37°C for 24 h. Photographs were taken at 0 h and 24 h, and the migratioin distance was measured by Image J (NCBI). The cells' migration rate was obtained by counting three fields per area and was represented as the average of six independent experiments done over multiple days.

Invasion assay

Invasion of A549 cells was measured using the BD BioCoatTM BD Matrigel TM Invasion Chamber (BD Bioscience, NJ, USA) according to the manufacturer's protocol. The medium in the lower chamber contained 5% fetal calf serum which acts as a source of chemo-attractants (in the absence of FCS in the upper chamber). Cells were suspended in serum-free medium containing Neratinib (2.5 μmol/L for U2-OS and 3 μmol/L for 143B cells) and added to the upper chambers simultaneously (2×10^3 cells in 0.1 ml). Cells that passed through the Matrigel-coated membrane were stained with Diff-Quik (Sysmex, Kobe, Japan) and photographed (×400). Photographs were taken at 24 h, and cell counting was measured by Image J (NCBI). The values for the invasion were obtained by counting three fields per membrane and represented as the average of six independent experiments done over multiple days.

Western blot analysis

Total protein from the OS cells was extracted using RIPA lysis buffer containing 6 μg/ml PMSF. Protein concentration was determined by Bradford assay. Equal amounts of protein were electrophoresed by 8% SDS-PAGE and transferred onto a pure Nitrocellulose blotting membrane (0.22 ml). Membranes were blocked with 5% skim milk for 1 h at room temperature, then blocked with primary antibodies (mouse rabbit anti-human p-HER2, PI3K, AKT, p-Akt, 1:2,000; mouse anti-human HER2, 1:2,000; β-actin IgG, 1:5,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) overnight at 4°C. Membranes were washed before incubated with appropriate peroxidase-conjugated secondary antibodies (anti-rabbit, anti-mouse, 1:5,000; Santa Cruz Biotechnology, Inc.).The immune complexes were detected with pro-light HRP Kit (TIAN GEN, China). GAPDH (1:2,000, Santa Cruz, Sh-

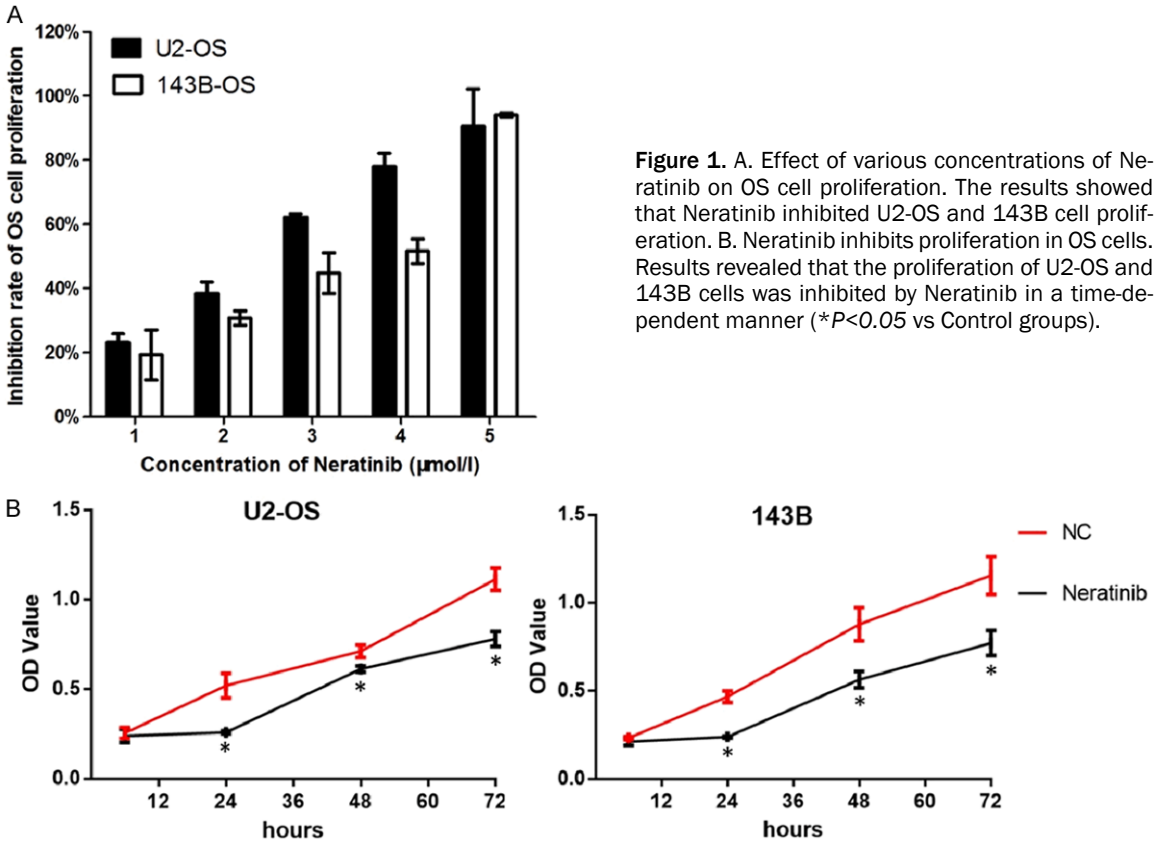

Figure 1. A. Effect of various concentrations of Neratinib on OS cell proliferation. The results showed that Neratinib inhibited U2-OS and 143B cell proliferation. B. Neratinib inhibits proliferation in OS cells. Results revealed that the proliferation of U2-OS and 143B cells was inhibited by Neratinib in a time-dependent manner (*$P<0.05$ vs Control groups).

anghai, China) protein expression was used as a normalization control for protein loading. All experiments were repeated by six times over multiple days.

Quantitative real-time PCR (qRT-PCR)

U2-OS and 143B cells in the exponential growth phase were treated with Neratinib (2.5 μmol/L for U2-OS and 3 μmol/L for 143B cells) for 24 h. Total RNA was extracted using Trizol (Invitrogen) method. HER2 expression level was evaluated by quantificational real time-PCR, and GAPDH was used as the endogenous reference genes. All amplifications were performed in the final reaction mixture (20 μl). Primer sequences used to amplify the containing were as follows: HER2 sense 5'-GGCACAGTCTACAAGGGCAT-3; HER2 antisense 5'-AGGGCA TAAGCTGTGTCACC-3'; β-actin sense 5'-TCACCCACACTGTGCCATCATCGA-3', β-actin antisense 5'-CAGCGGAACCGCTCATTGCCAATGG-3'. The amplification reaction was performed using MJ real-time PCR (Bio-Rad, Hercules, CA, USA) for 40 cycles. The amplification reaction was performed using StepOne Real-Time PCR System for 40 cycles. Relative expression was calculated using the 2-ΔΔCt method.

Statistical analysis

All measurement data are presented as the mean ± standard deviation. The differences in invasion and migration capabilities between the cells treated with and without Neratinib were evaluated with independent-sample t-tests. $P<0.05$ was considered to indicate a statistically significant difference. All analyses were performed using SPSS statistical software version 13.0 (SPSS, Inc. Chicago, IL, USA).

Results

Effect of Neratinib on OS cell proliferation

The effect of Neratinib on the proliferation of the U2-OS and 143B cell lines was investigated using CCK-8 assays. The results showed that the proliferation of U2-OS and 143B cells was inhibited by Neratinib in a dose-dependent manner at 24 h, and the proliferation of U2-OS and 143B cells was inhibited by Neratinib in a

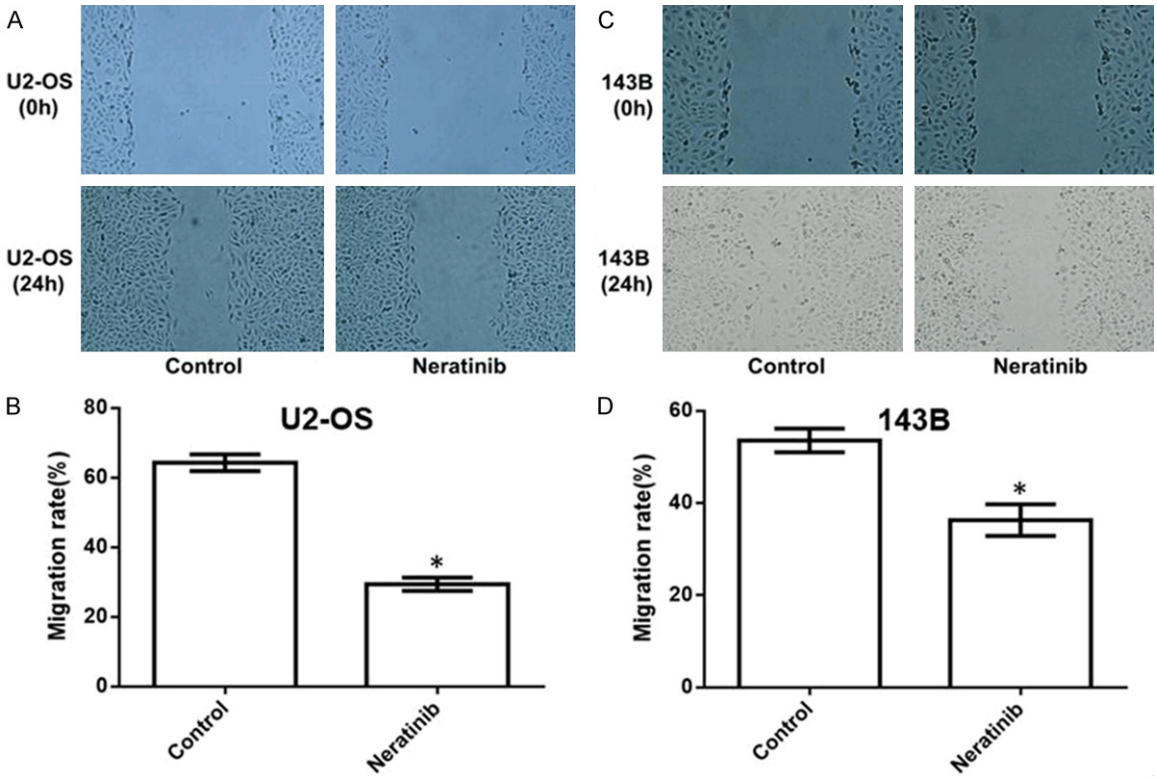

Figure 2. Neratinib inhibited U2-OS and 143B cell migration *in vitro*. The migration rate was significantly lower in cells treated with Neratinib than those cells without treated (*$P<0.05$ vs Control groups).

time-dependent manner (**Figure 1**). The IC50 values for Neratinib in U2-OS and 143B cells at 24 h were 2.431 and 2.966 μmol/L. A concentration of 2.5 μmol/L and 3.0 μmol/L Neratinib was chosen for treatment of U2-OS and 143B cells respectively in the following assays.

Effect of Neratinib on OS cells migration

To examine the effect of Neratinib on OS cell migration, the migration capability was assessed with the wound healing assay. The wound healing assays showed that the migrated rates of U2-OS (**Figure 2A**, **2B**) and 143B (**Figure 2C**, **2D**) cells treated with Neratinib were significantly lower than in those cells without treated. These data indicated that Neratinib could suppress the migratory ability of OS cells *in vitro*.

Effect of Neratinib on OS cell invasion

To examine the effect of Neratinib on U2-OS and 143B cell invasion, the invasion capability was assessed with the Transwell invasion assay. In the Transwell invasion assay, the invasion of the cells treated with Neratinib was significantly inhibited compared with that in the untreated cells (**Figure 3**). This suggests that Neratinib suppresses U2-OS and 143B cell invasion *in vitro.*

Neratinib suppresses the activity of the HER2 and PI3K/AKT signaling pathway in OS cells

To investigate the effect of Neratinib on the activity of the HER2 and PI3K/Akt signaling pathway, the protein expression levels of p-HER2 (Tyr877), PI3K, p-Akt, and Akt were detected. The results showed that the protein expression levels of p-HER2, PI3K, p-Akt except for Akt were significantly decreased in the cells treated with Neratinib when compared with these levels in the untreated cells (**Figure 4**). This suggests that Neratinib suppresses the activity of HER2 and PI3K/Akt in U2-OS and 143B cells *in vitro*.

Discussion

In the 1960s, several studies had already revealed that the 5-year survival rates of osteosarcoma were below 50%. But following the discovery of effective chemotherapy in the past

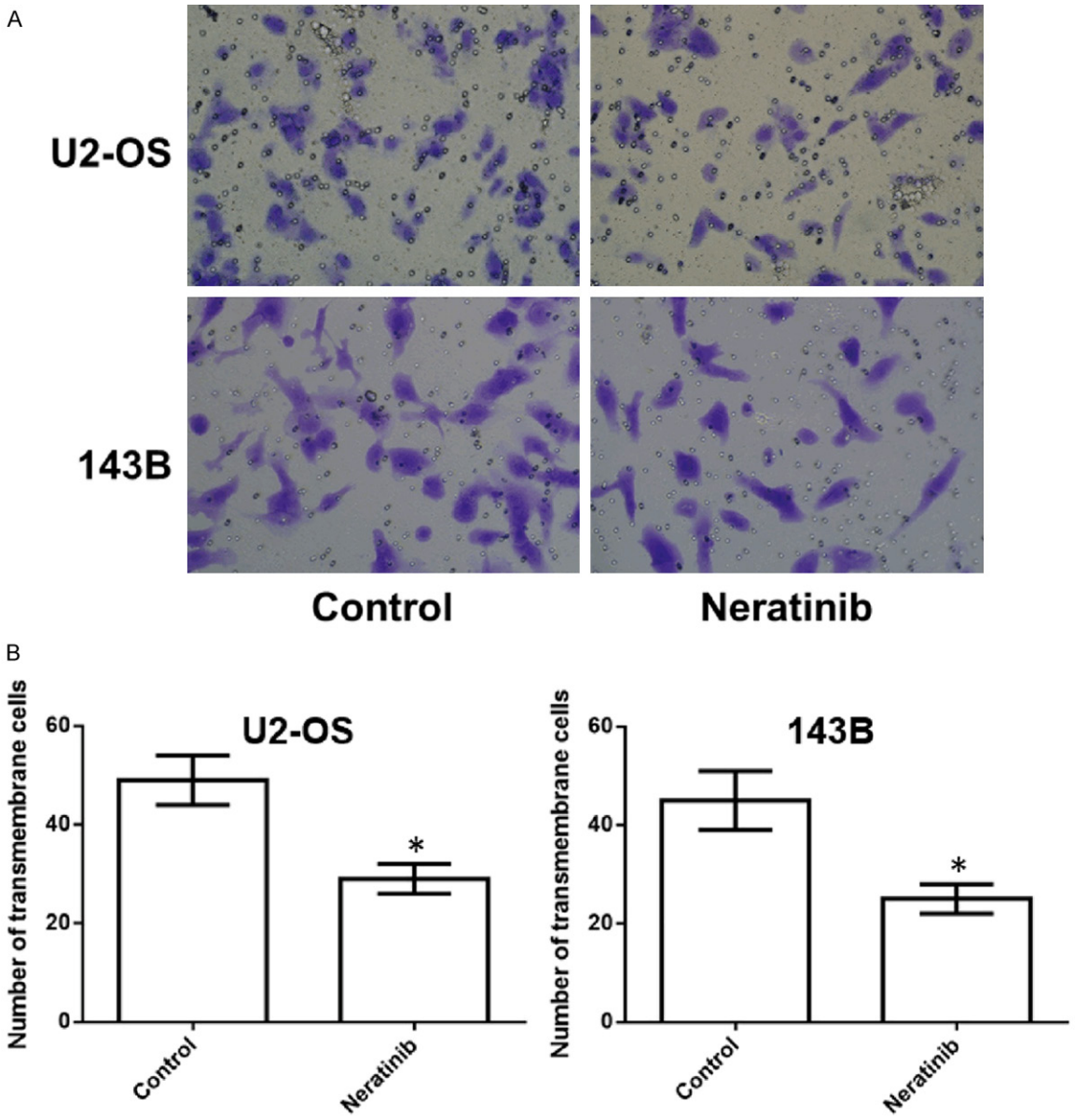

Figure 3. Transwell invasion assay was used to evaluate the effect of Neratinib on OS cell invasion. In U2-OS and 143B cell lines, the number of transmembrane cells in the Neratinib-treated group was significantly fewer than the number of the cells in control group (*$P<0.05$ vs Control groups).

60 years, the 5-year survival rates for patients treated with multi-drug chemotherapy and aggressive local control have been reported to be 55-80% [12]. However, a single chemotherapy regimen may be unable to eliminate all OS cells due to intrinsic or acquired drug resistance, which is the most common reason for tumor recurrence and resultant poor clinical outcomes [13]. Therefore, it is imperative to find out new drugs with improved chemotherapeutic effects for the treatment of OS.

Neratinib, a tyrosine kinase inhibitor, takes advantage of some features of the epidermal growth factor receptor tyrosine kinase ATP binding site to obtain strong selectivity. It binds to the ATP binding site of the tyrosine kinase region by hydrogen bonds, which prevents ATP from binding to the tyrosine kinase region, and inhibits tyrosine kinase autophosphorylation and activation.

HER2, a proto-oncogene belongs to type I receptor tyrosine kinase (RTK) ErbB subfamily in the family, and is located on chromosome 17q21. Studies have confirmed that HER2 activates self-phosphorylation without extracellular ligand and encourages the invasion and

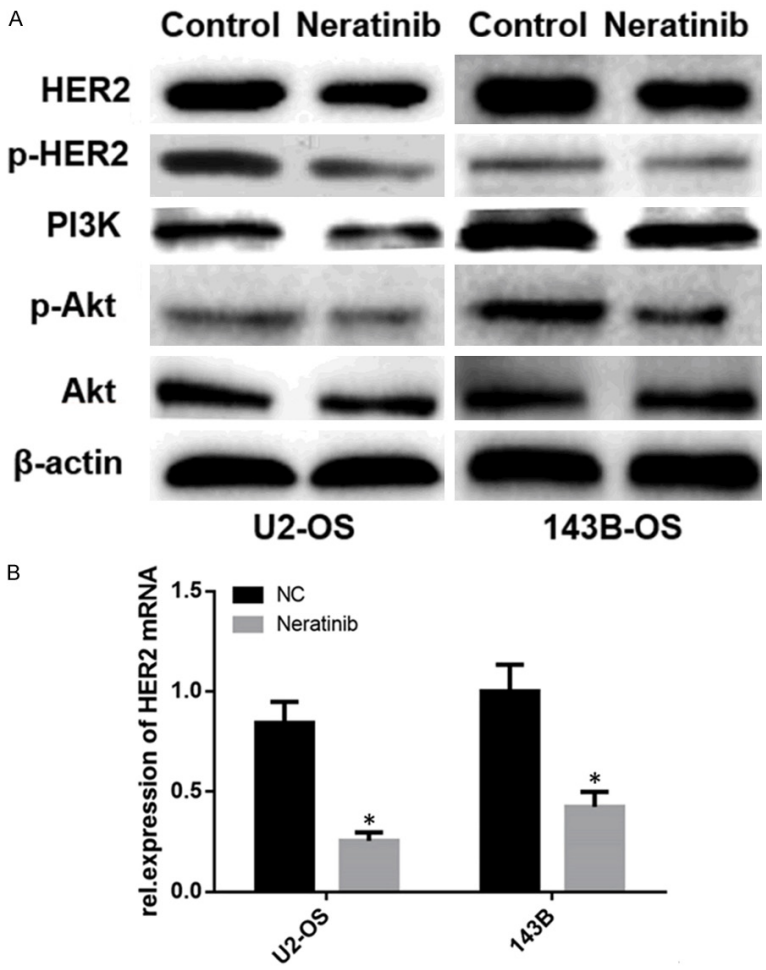

Figure 4. A. Western blot analysis of the expression levels of p-HER2, HER2, PI3K, p-Akt and Akt in OS cells. Results showed that the expression levels of all proteins except for AKT were significantly inhibited by Neratinib. B. The expression of HER2 mRNA was measured by qRT-PCR. It revealed that the HER2 mRNA level was inhibited by Neratinib in U2-OS and 143B cells *in vitro* (**P<0.05* vs Control groups).

metastasis of osteosarcoma cells by PI3K/Akt pathway [14]. Moreover, Neratinib inhibits activation of EGFR/HER2, thus inhibiting cell proliferation [11]. However, the antitumor effect of Neratinib and its mechanism in osteosarcoma needs further study. Previous studies have demonstrated that HER2 expression modulates the activation of the PI3K/Akt pathway in OS cells [8]. It is therefore hypothesized that Neratinib may inhibit the expression of HER2 by PI3K/Akt signaling pathway, resulting in inhibition of the malignant phenotype in OS cells. To confirm these hypotheses, U2-OS and 143B cells were treated with Neratinib. Interestingly, our results revealed the protein of HER2, p-HER2, PI3K and p-Akt were significantly inhibited in cells treated with Neratinib when compared to the negative control group. These indicated that Neratinib inhibits the activity of HER2-PI3K/Akt signaling pathway in U2-OS and 143B cells. Meanwhile, the cells migration, invasion and proliferation abilities were declined in cells treated with Neratinib.

In summary, our findings demonstrated that Neratinib could modulate HER2-PI3K/Akt signaling pathway, resulting in decline of the malignant phenotype of osteosarcoma cells in vitro. It provides a new idea for the prevention and treatment of osteosarcoma. However, the evolution, invasion and migration of tumors are closely related to the microenvironment of the tumor, further studies *in vivo* are needed to confirm the effect of Neratinib on the malignant phenotype of osteosarcoma cells mediated by HER2.

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Disclosure of conflict of interest

None.

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